Effects of Insecticides, Fungicides, and Adjuvant Combinations on Honey Bee Brood Development

Undergraduate Research Distinction Thesis

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ABSTRACT

California's almond industry is responsible for producing the majority of the world's almonds, and America's honey bee colonies provide valuable pollination services to these orchards. Various fungicides, insecticides, and adjuvants are used to minimize crop damage from various diseases and pests. Recent research has demonstrated that these preventative treatments negatively impact colony health, especially by increasing brood mortality. This is problematic for beekeepers and the almond industry who rely on strong, healthy colonies.

In this study honey bee larvae were reared to adulthood, feeding them a treated diet simulating exposure to commonly used chemicals. First, an organosilicone-based nonionic methylated seed oil known as Dyne-Amic was added in various concentrations to the royal jelly diet to create a dose-response curve. The LC50 was determined to be 1.9x lower than the recommended field application rate for Dyne-Amic. Second, a sublethal dose of Dyne-Amic was combined with sublethal doses of an insecticide, a fungicide, or both to observe any compounding effects. Preliminary results showed no increase in mortality resulting from a mixture of the insecticide, fungicide, and Dyne-Amic. Only sublethal concentrations were used in these trials, and ongoing trials will expand the concentration exposure.

The results of this research are important for the almond growers in California, as well as any agricultural practice that utilizes combinations of pesticides, fungicides, and adjuvants. Particularly for almond growers, understanding these chemical interactions can help improve the efficiency of bee pollination services and protect honey bee health. Product label application instructions may be refined for common insecticides, fungicides, and the surfactant Dyne-Amic as a result of these findings.

INTRODUCTION

Honey bees, *Apis mellifera,* are highly valued in the booming almond industry because they serve as vital pollinators needed to cross-pollinate almond flowers. In the United States, California is the leader in the almond industry, producing approximately 80% of the world's almonds.[1] It is estimated that over 2 million of America's commercial honey bee colonies are transported annually to pollinate California's almond flowers. The pollinating season begins in mid-February and continues through mid-March, as the almond buds begin to open in anticipation of pollination.[1] During the almond season, various insecticides, fungicides, and adjuvants are used to combat common pests and diseases such as brown rot, leaf blight, hull rot, and the peach twig borer.[4] Propiconazole (found in Tilt) and iprodione (found in Rovral) are common active ingredients in major fungicide products. Methoxyfenozide (found in Intrepid 2F), diflubenzuron (found in Dimilin 2L), and chlorantraniliprole (found in Altacor) are all common active ingredients in major insecticide products. The practice of utilizing insecticides and fungicides is to minimize crop damage and maximize crop yield. Adjuvants are products designed to enhance the efficacy of insecticides, herbicides, and fungicides through various mechanisms such as decreasing surface

tension, modifying pH, slowing drift, and acting as a defoaming agent. [5] Adjuvants are considered to be biologically inert by the EPA and are therefore exempt from food tolerance residues and are not monitored at a federal level.[3] Surfactants are a class of adjuvants that specifically help reduce surface tension to maximize penetration of the pesticide. Organosilicone-based surfactants (OSS) are considered the gold standard in the adjuvant industry because they are highly effective.[5]

This study focuses on the most widely used surfactant known as Dyne-Amic, an organosilicone-based, nonionic methylated seed oil.[6] Research has demonstrated that OSS have a toxic effect on honey bees.[6] Reports from 2014 and 2015 indicated that beekeepers observed healthy adult workers but failing brood in subsequent weeks following almond bloom and pollination events, indicating that various chemical cocktail applications before and during almond bloom may be contributing to the rise in observed honey bee mortality. Furthermore, research has demonstrated that OSS exposure coupled with normal encounters of environmental viruses increases Black Queen Cell Virus (BQCV) titers.3 Contrary to popular belief, this data demonstrates that organosilicone spray adjuvants are not biologically inert, and in fact are capable of increasing the pathogenicity of viruses.3

This is problematic for beekeepers and the almond industry who rely on strong, healthy colonies. Therefore, this research has two objectives: to understand the impact of exposing colony brood to the surfactant Dyne-Amic and furthermore, if any synergistic interactions occur when Dyne-Amic is combined with commonly used insecticides and fungicides. Ultimately, the results of this research may result in updated product label guidelines for these commonly used products to more appropriately reflect the biological activity of surfactants.

MATERIALS & METHODS

The protocol for *in vitro* rearing of honey bee workers by Schmehl et al. (2016) was used to perform larval rearing bioassays to simulate larval exposure to propiconazole, iprodione, methoxyfenozide, diflubenzuron, chlorantraniliprole, and Dyne-Amic in various concentrations and combinations (i.e. a fungicide and an insecticide, or a fungicide, insecticide, and Dyne-Amic) (**Figure 2**). Brood frames containing larvae approximately less than 24 hours old were removed from either the Waterman Farm or Aronoff Laboratory colonies in Columbus, Ohio and grafted into sterile, 48-well tissue culture plates containing sterile, brown plastic cell cups.

Care was taken to ensure that larvae were transferred in the same orientation from which they originated in their brood frame cell, with spiracles facing upwards. This minimized the incidence of larvae drowning in the 20uL of larval diet A consisting of royal jelly, D-fructose, D-glucose, yeast extract, autoclaved water, and royal jelly. These culture plates were then incubated for 48 hrs in a desiccator at 94% RH and 35 C. 48 hrs post grafting, the larvae were fed 20uL of larval diet B consisting of a higher percentage of sugars and returned to the dessicator. 72 hrs post grafting, the larvae are assessed to determine viability. Submerged, dead, or undersized larvae that

did not consume all of their diet were removed and appropriately discarded (**Figure 3**). Remaining, healthy larvae were then divided into equal trials (i.e. 16 in one row, 16 in another row, etc.) and fed 30uL of larval diet C consisting of an even higher percentage of sugars and various concentrations of either the surfactant Dyne-Amic, an insecticide, or a fungicide, or a combination of all three (**Table 1**). Each trial was then appropriately labeled according to the combination of pesticide and adjuvant added, and the larvae were returned to the desiccator. 96 hrs post grafting, the larvae were fed 40uL of diet C, and at 120 hrs post grafting, the larvae were fed 50uL of diet C. After each feeding, mortality was assessed by presence of melanization, submersion in diet, and lack of spiracle movement. If any of these observations were made, the larva was considered dead, and mortality was recorded. Dead larvae were not removed from the tissue culture plate until it was time to transfer larvae to the pupation plates.

Pupation plates consisted of 48-well tissue culture plates lined with sterilized KimWipes approximately 1.5”x0.5”. At 144 hrs post grafting, the larvae were removed from the dessicator, and a Chinese grafting tool was used to transfer larvae from the larval plates to the pupation plates. Only larvae that had consumed all of the diet and demonstrated spiracle movement were deemed alive and healthy and were transferred to the pupation plates. Pupation plates were then placed in a pupal desiccator at 75% RH and 35 C. Mortality was checked every other day up until day 18 when the pupae were checked daily. Adult emergence began as early as day 18 and continued until day 20. Successful and failed emergence were both noted for each trial, and the results were used to plot a dose-response curve for Dyne-Amic as well as identify if any synergistic effects, such as increased mortality rate, occurred as a result of interactions between pesticide and adjuvant combinations. Trials that contained a variation of combinations of insecticides, fungicides, and Dyne-Amic were conducted using established sublethal concentrations (**Table 2**).

RESULTS

Dyne-Amic was tested at various concentrations to collect data for a dose-response curve and exhibited increasing lethal effects as concentration increased that ultimately decreased total adult emergence when compared to the control groups (**Figure 4**). This indicates that the presence of adjuvants may be contributing to brood mortality in honey bee colonies, demonstrating that Dyne-amic can be biologically active. The recommended field rate application for Dyne-Amic is 3 fluid oz/gallon or 2.34%. The LC50 for Dyne-Amic was determined to be 1.2% based on 15 different trials containing on average, 14 bees per test. Therefore, current recommended field applications are 1.9x higher than the LC50 determined in these trials.

The results of this study indicate that Dyne-Amic alone is capable of killing larvae and pupae indicating that it may not be biologically inert as previously indicated. The exact mechanism is unknown; however, failure to successfully pupate or eclose suggests a maturation pathway is inhibited. Further research is needed to more fully understand OSS’s mode of action. Preliminary results did not demonstrate an increase in mortality rate when Dyne-Amic was combined with the

fungicides and insecticides (**Figure 4**). This may or may not be attributed to using sublethal concentrations, or the progression of the season from June-September 2018. Future trials will identify whether or not these factors did contribute to the preliminary findings.

Additionally, this study was performed in a laboratory environment by artificially rearing honey bee brood. Actual concentrations that foraging bees are exposed to may depend on the weather, temperature, time of day, and amount of time between spraying and foraging. These factors are all field-related, and future investigation is warranted to expand laboratory knowledge to field exposure. Furthermore, trophallaxis complicates the pesticide concentration as foragers return to the colony and distribute collected pollen and nectar to nurse bees. Nurse bees then feed larvae, so whether bioaccumulation occurs throughout the trophallaxis process is still under investigation.

Regardless, this research provides a valuable foundation for future studies investigating this important topic. Gaining a more thorough understanding of the situation will help both beekeepers and almond growers alike in protecting honey bee colonies and maximizing agricultural output. Furthermore, the environmental impact of studying this model organism is incredibly useful for broader research evaluating pesticide impact on non-target organisms.

CONCLUSIONS

These trials made a novel discovery that an adjuvant alone is capable of inducing brood mortality. This data may revise the recommended application rates on adjuvants, as well as alter spraying protocols during almond bloom. Further research will expand the knowledge on possible synergistic effects of fungicides, insecticides, and adjuvant combinations, in addition to exploring actual field exposure that honey bees encounter. The long-term goal of this research is to protect beekeeper colonies and maximize almond production by thoroughly understanding how these combinations affect honey bee colonies. Once these effects are known, appropriate procedures can be modified to ensure healthy colonies while still obtaining fruitful almond harvests.

ACKNOWLEDGEMENTS

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FIGURES

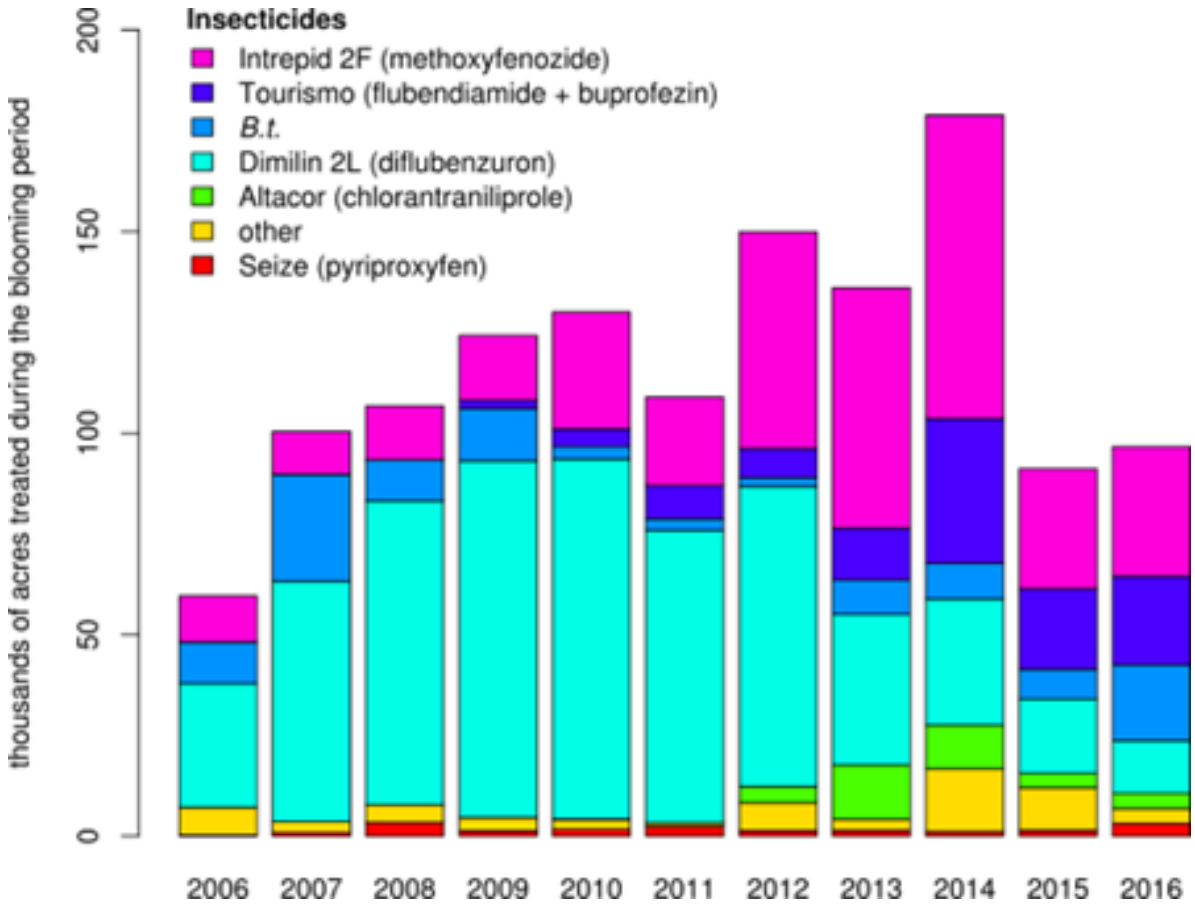

Figure 1A. Annual evaluation of insecticides that are commonly used and applied during almond bloom from February 15 – March 15 in California

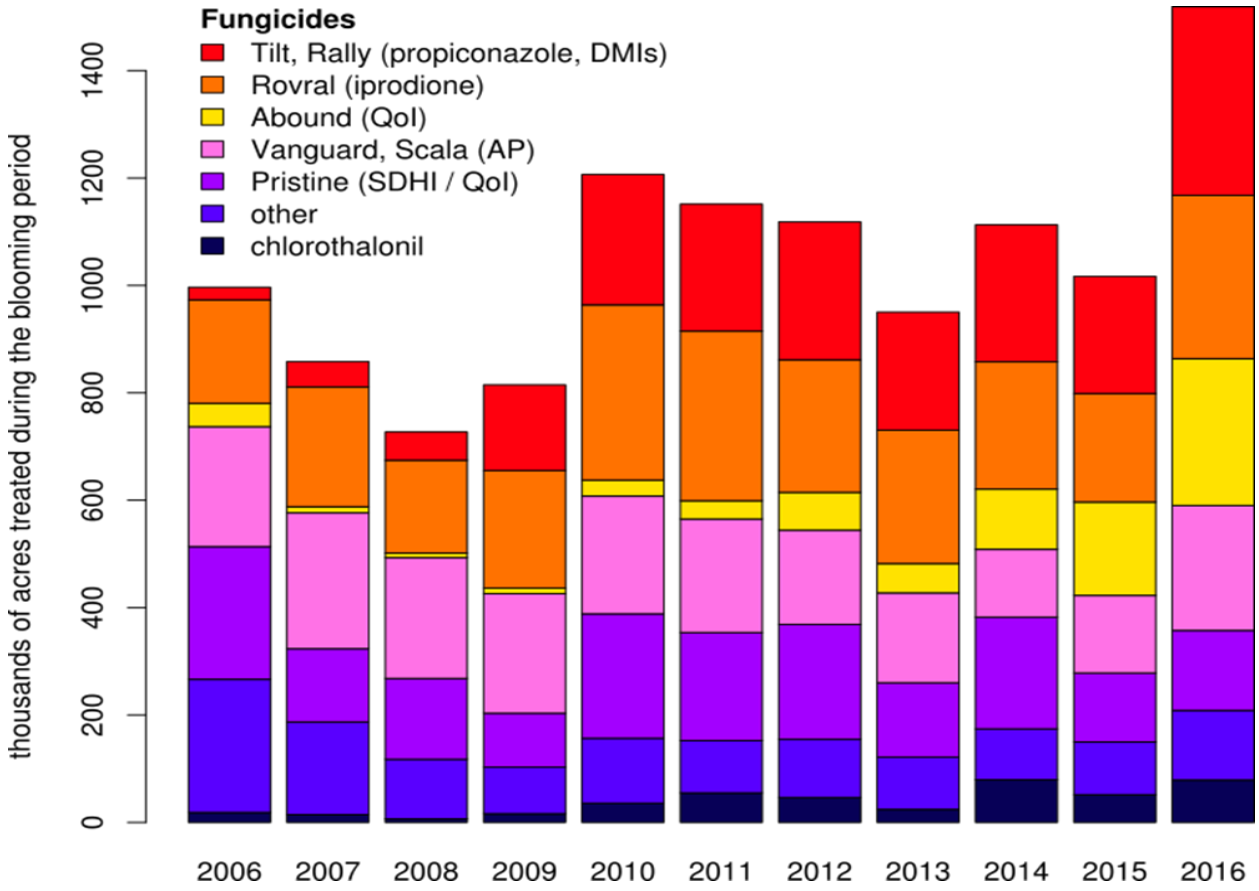

Figure 1B. Annual evaluation of fungicides that are commonly used and applied during almond bloom from February 15 – March 15 in California

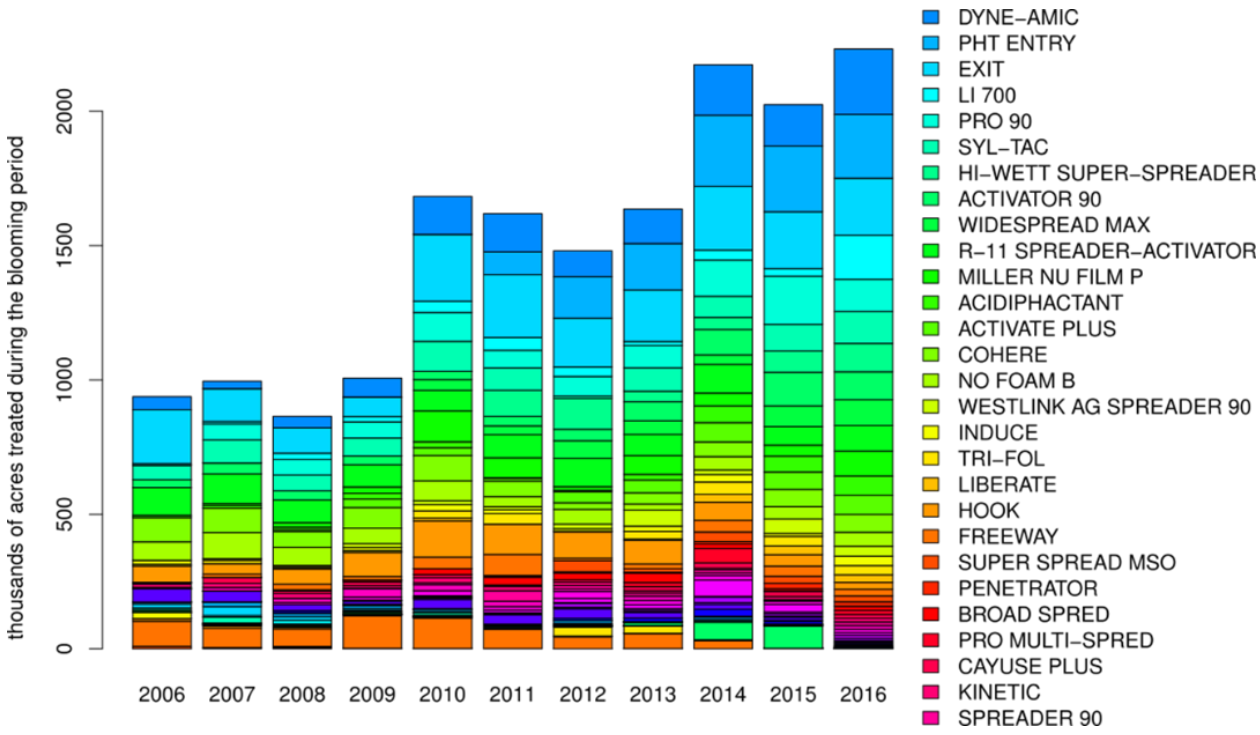

Figure 1C. Annual evaluation of adjuvants that are commonly used and applied during almond bloom from February 15 – March 15 in California

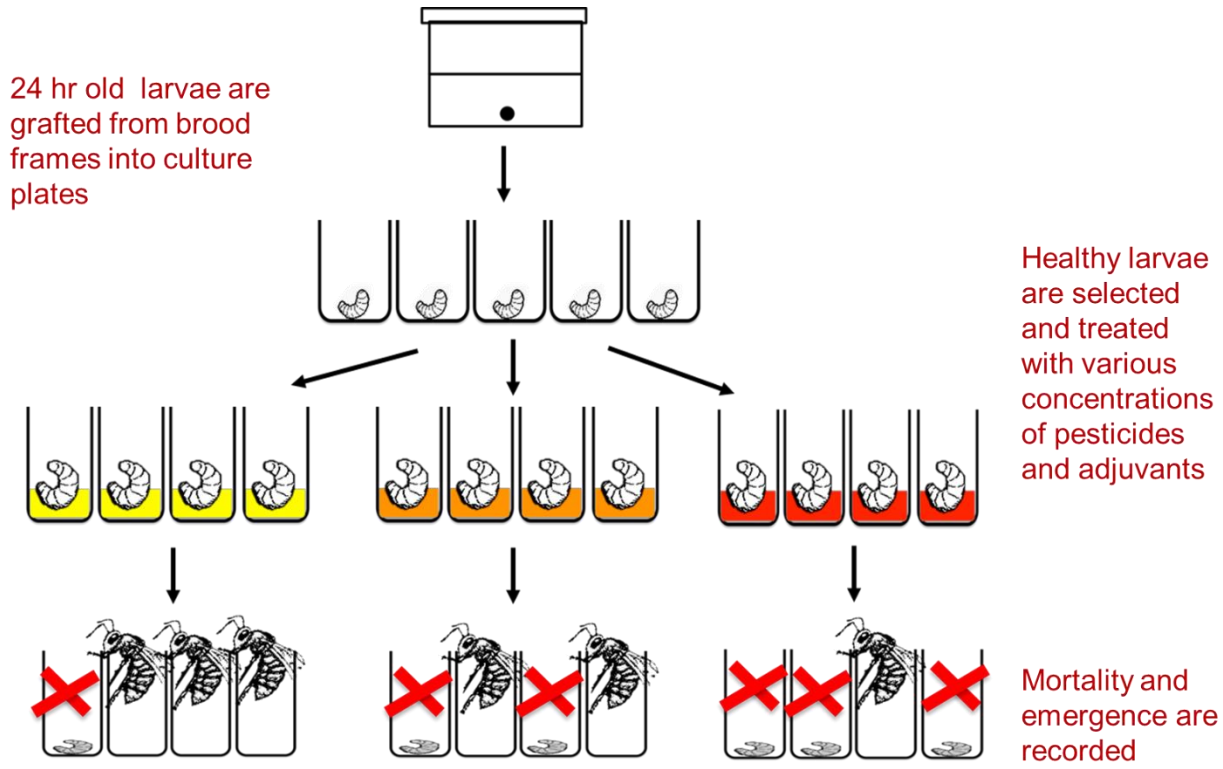

Figure 2. Pictorial summary highlighting the *in vitro* larval rearing process according to the Schmehl et al. protocol

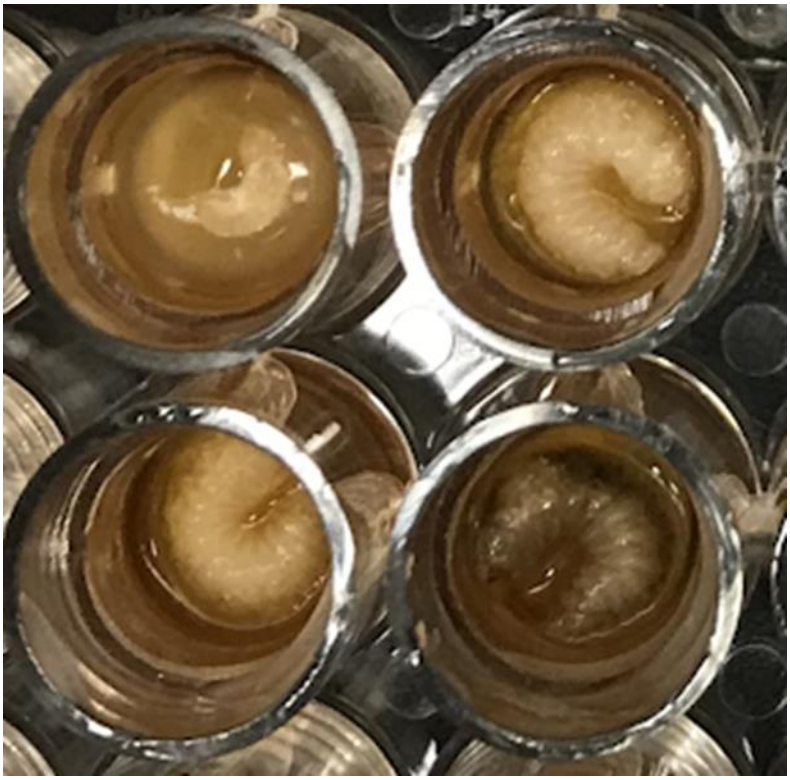

Figure 3. Examples of Observed Larval Mortality
A = sunken larvae; B = unfinished diet; C = melanization

Dyne-Amic	Concentrations
0.17	0.83
0.25	1.00
0.33	1.17
0.42	1.33
0.50	1.50
0.67	1.75
0.75	2.00

Table 1. Dyne-Amic concentrations tested (% of the total diet)

Pesticide Used	Sub-lethal Concentrations
Chlorantraniliprole	0.83
Diflubenzuron	0.17, 0.33
Methoxyfenozide	1.00
Iprodione	1.00
Propiconazole	1.00
Chlorantraniliprole + Propiconazole	0.67. 0.83, 1.00
Chlorantraniliprole + Iprodione	1.00

Insecticide

Fungicide

Table 2. Sub-lethal pesticide concentrates tested with Dyne-Amic 0.33% (% of the total diet)

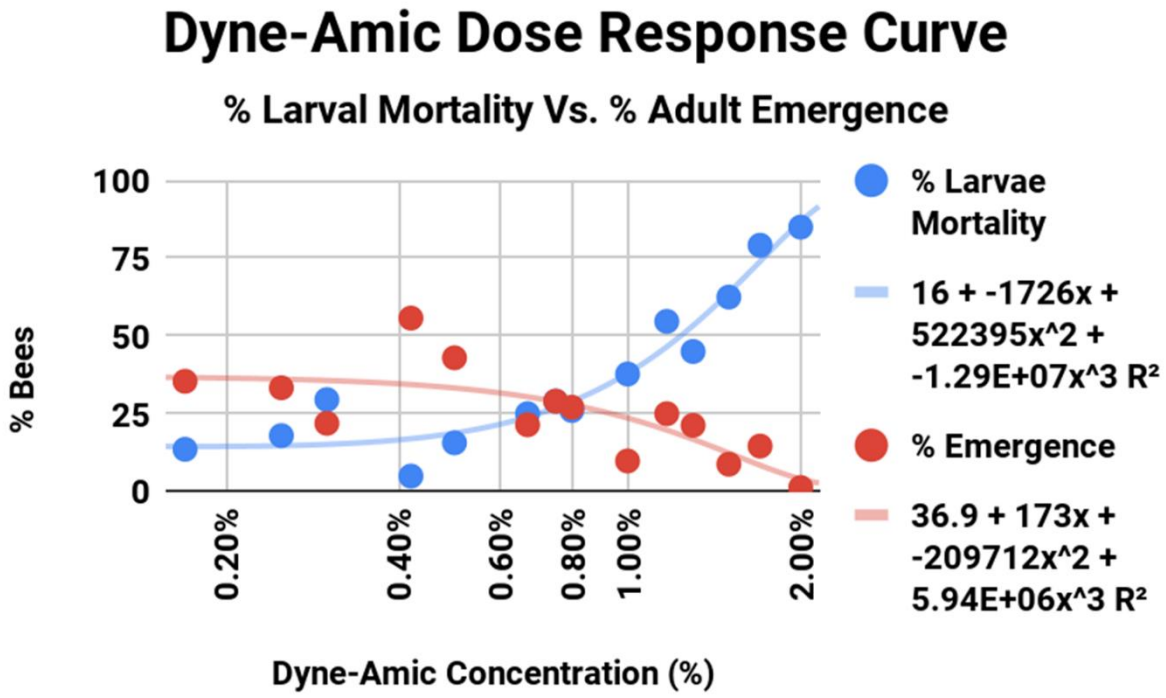

Figure 4. Dyne-Amic dose response curve demonstrating the impact of increasing Dyne-Amic concentration on larval mortality and subsequent adult emergence

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